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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant:	Roland Valdes, Jr. et al.	Examiner:	Ulrike Winkler
Serial No.:	09/503,559	Group Art Unit:	1648
Filed:	February 11, 2000	Docket:	1160.003US1
Title:	DIHYDROOUABAIN-LIKE FACTOR AND DIAGNOSTIC & THERAPEUTIC COMPOSITIONS AND METHODS		

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DECLARATION OF DR. ROLAND VALDES, JR. UNDER 37 C.F.R. § 1.132

1. I, Roland Valdes, Jr., am one of the co-inventors of the above-identified patent application and am currently a Professor in the Department of Pathology and Laboratory Medicine at The University of Louisville in Louisville, Kentucky.
2. Physical properties related to molecular polarity and solubility can be distinguished by HPLC analysis. Thus, under identical experimental condition, a difference in chromatographic mobility is demonstrative of structural differences existing between the entities chromatographed.
3. In order to compare plant-derived dho and Dh-OLF, I performed the following experiments. Plant dihydroouabain (dho) and the mammalian Dihydro-OLF was isolated using standard purification techniques. The preparations were chromatographed both separately and mixed together under identical experimental conditions. The eluted HPLC fractions were measured using an antibody assay specific for the dihydro compounds.
4. Pure Dh-OLF was obtained from mammalian adrenal cortex (Qazzaz et al., Endocrinology, 2000;141(9):3200-3209) and pure Dho-B was obtained from HPLC separation of dihydroouabain commercial preparation (Qazzaz et al., Biochem Biophys Acta, 1999:1472:486-497). These experiments were performed with the purified intact molecules of dho and Dh-OLF and also after removal of the sugar moiety from each by hydrolysis. This approach tested both the intact molecule and the genin-form of each compound.
5. To remove the rahmonse moiety the parent molecules (Dh-OLF and Dho-B) were treated individually with 1% SSA for 45 seconds and the acid was immediately removed using a small C-

18 reversed-phase Sep-Pak solid-phase extraction cartridge column previously wetted with acetonitrile (AcN) and rinsed with deionized water. The Sep-Pak columns were eluted with 2 ml of 100% acetonitrile. To remove the AcN, the Sep-Pak eluents were evaporated to dryness in a Savant Speed Vac, dissolved the residue in 1 ml deionized water, and filtered the solution through a Whatman 0.22 μ m PVDF filter in preparation for HPLC. The compounds were mixed together and co-injected on HPLC using an isocratic 10% CH₃CN mobile phase.

6. The two molecules, dho-B and Dh-OLF, separated by almost two minutes. Similarly, the genin compounds (aglycones, without sugar molecules) of both parents when mixed and injected on HPLC also separated by approximately 2 minutes on the same isocratic HPLC mode. The parent compounds, Dh-OLF and Dho-B eluted at 26 and 28, respectively. Their genin components (Dh-OLF-genin and dihydroouabain-B-genin) eluted at 18 and 20 minutes, respectively (See, Attached Figure).

7. The separation of plant dihydroouabain from mammalian Dh-OLF and of dho-genin from Dh-OLF-genin by HPLC demonstrates conclusively that a structural difference exists between the plant dho and the Dh-OLF mammalian compounds.

8. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that the statements are made with the knowledge that willful false statement and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

3/28/03

Date

RJ

Roland Valdes, Jr.